The proteasome inhibitor MG132 promotes accumulation of the steroidogenic acute regulatory protein (StAR) and steroidogenesis

Kimihisa Tajima[a], Svetlana Babich[a], Yoshio Yoshida[a], Ada Dantes[a], Jerome F. Strauss III[b], Abraham Amsterdam[a,*]

[a] *Department of Molecular Cell Biology, The Weizmann Institute of Science, 76100 Rehovot, Israel*
[b] *Center for Research on Reproduction and Women's Health, University of Pennsylvania, Philadelphia, PA 19104, USA*

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Abstract StAR, a protein synthesized in the cytoplasm and subsequently imported into mitochondria, regulates the rate-determining step in steroidogenesis, the transport of cholesterol from the outer to the inner mitochondrial membrane. The active form of StAR is the 37 kDa pre-protein, which has a short half-life. To determine whether proteasomes participate in the turnover of StAR, we incubated primary cultures of preovulatory rat granulosa cells and immortalized human granulosa cells in the presence of MG132, a specific inhibitor to proteasome catalysis. This treatment caused accumulation of StAR in unstimulated cells. Moreover, incubation of the cells with MG132 in the presence of forskolin (FK), luteinizing hormone/chorionic gonadotropin or follicular stimulating hormone augmented the accumulation of both the 37 kDa cytoplasmic protein and the 30 kDa mature mitochondrial protein, compared to cells incubated with FK or the gonadotropic hormones alone. Concomitantly, progesterone production was enhanced. In contrast no elevation in the 37 kDa StAR intracellular levels or progesterone production was observed following incubation of the cells with the cysteine protease inhibitor E-64. The increase of the 37 kDa StAR protein was evident after 15 min and 30 min of incubation with MG132 (143% and 187% of control values, respectively) with no significant elevation of the 30 kDa protein. Accumulation of the intermediate mitochondrial 32 kDa protein was evident after 1–2 h and the accumulation of the 30 kDa protein was evident only after 4 h of incubation with MG132. In contrast, no elevation in adrenodoxin, a component of the cytochrome P450scc enzyme system, was found. These data suggest that StAR protein is either directly or indirectly degraded by the proteasome which may explain, in part, its short half-life. Moreover, it seems that the cytosolic 37 kDa protein, which is responsible for the steroidogenic activity of StAR, is the primary proteasomal substrate and that the inhibition of its degradation by MG132 causes the up-regulation of progesterone production.

Key words: StAR degradation; MG132; Proteasome; Ovary; Steroidogenesis

1. Introduction

The steroidogenic acute regulatory protein (StAR) is synthesized as a 37 kDa protein in the cytoplasm of steroidogenic cells and is then rapidly transported into mitochondria where it is cleaved, generating 32 kDa and 30 kDa proteins [1,2]. StAR controls the rate-limiting step in steroidogenesis, the transport of cholesterol from the outer to the inner mitochondrial membrane where the cytochrome P450scc enzyme system is located. The 37 kDa protein is believed to be the active form of StAR and it is thought that the C-terminus of the protein acts on the outer mitochondrial membrane to promote cholesterol translocation [3]. The import of the cytosolic pre-protein into the mitochondria is consequently thought to be the event that terminates StAR's steroidogenic activity. Because the 37 kDa pre-protein has a short half-life, it must be produced continuously if steroidogenesis is to be maintained. The up-regulation of StAR by gonadotropin/cAMP signaling has been studied in detail [2,4]. However, little is known about its degradation. Proteasomes catalyze the degradation of nuclear and cytoplasmic proteins and peptide hormones including NF-κB [5], p53 [6], ornithine decarboxylase [7] and gonadotropin releasing hormone [8]. Moreover, it was recently documented that sperm mitochondria are degraded by the ubiquitin–proteasome system following penetration of the sperm to the egg during fertilization [9]. Thus, proteasomes participate in the catabolism of proteins in a number of subcellular compartments. In the present work we demonstrate for the first time that inhibition of the proteolytic activity of the proteasome results in the accumulation of StAR, implicating the proteasome in StAR turnover.

2. Materials and methods

2.1. Reagents

MG132 was purchased from Calbiochem-Novabiochem Co. (La Jolla, CA, USA). Forskolin (FK, a potent activator of adenylate cyclase), E-64 (a cysteine protease inhibitor) and 4′,6-diamido-2-phenylindole hydrochloride (DAPI; for DNA staining) were purchased from Sigma Israel Chemical Ltd. (Rehovot, Israel). Antibodies to cytochrome *c* were purchased from BD PharMingen (San Diego, CA, USA). Human follicular stimulating hormone (hFSH) and human chorionic gonadotropin (hCG) were kindly provided by the NIH and Dr. Parlow.

2.2. Cell culture

Primary granulosa cells of preovulatory follicle were obtained from 25-day-old female rats treated with 15 IU PMSG [10]. Cells were

*Corresponding author. Fax: (972)-8-934 4125.
E-mail: abraham.amsterdam@weizmann.ac.il

Abbreviations: StAR, steroidogenic acute regulatory protein; FSH, follicular stimulating hormone; hCG, human chorionic gonadotropin; NPC1, Niemann–Pick C1; ADX, adrenodoxin

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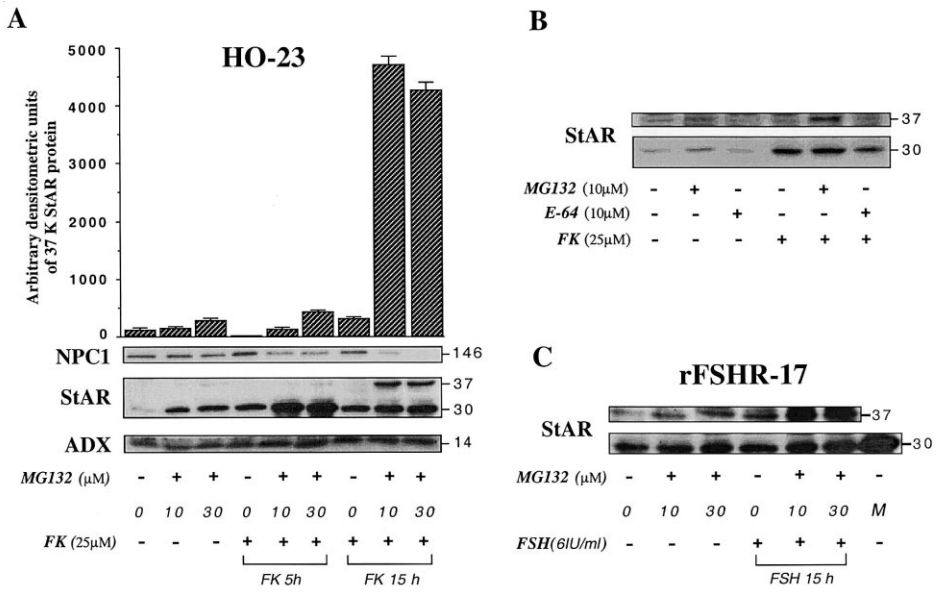

Fig. 1. Content of NPC1, StAR and ADX in immortalized human (HO-23: A,B) and rat (rFSHR-17: C) granulosa cells incubated at 37°C in the absence or presence of FK or FSH respectively for 15 h. The proteasome inhibitor MG132 or the cysteine protease inhibitor E-64 were added to the indicated cultures during the last 5 h of incubation. Cell lysates were separated by acrylamide gel electrophoresis, transferred to nitrocellulose membrane and reacted with the appropriate antibodies. M, enriched fraction of rat granulosa cell mitochondria.

plated in Dulbecco's modified Eagle's medium (DMEM):F12 (1:1) containing 5% fetal calf serum (FCS), on plastic tissue culture dishes. Cells were maintained at 37°C in 5% CO_2 in humidified incubators.

2.3. Establishment of human granulosa cell lines

The human granulosa cell line HO-23 was established by triple transfection of primary human granulosa cells obtained from an in vitro fertilization program, with SV40 DNA, Ha-ras oncogene, and a temperature sensitive mutant of p53 (p53Val135) as described [11]. The rat FSH responsive cell line (rFSHR-17) was established by triple transfection of preovulatory granulosa cells with SV40 DNA, Ha-ras oncogene, and FSH receptor plasmid [12]. Cells were maintained in DMEM/F12 (1:1) containing 5% FCS, at 37°C in the presence of 5% CO_2 in a humidified incubator.

2.4. Biochemical assays

Progesterone measurement. Progesterone accumulated in the culture medium was determined by radioimmunoassay [13,14]. Antibodies to progesterone were kindly provided by Dr. F. Kohen (The Weizmann Institute of Science, Rehovot, Israel).

Protein assay. Protein was quantified by the Bradford method [15].

2.5. Western blot analysis

Western blot analysis was carried out as described [11]. Samples containing equal amounts of protein (30 μg) were separated by 12% (to detect StAR and Niemann–Pick C1 protein (NPC1) [16]) or 15% (to detect adrenodoxin (ADX)) acrylamide SDS–PAGE. Anti-human ADX polyclonal antibodies were kindly provided by Dr. W.L. Miller (University of California, San Francisco, CA, USA). Anti-NPC1 N-terminus polyclonal antiserum was used to detect NPC1 [17].

2.6. Microscopy

Phase contrast and immunofluorescent microscopy was performed using a Zeiss Axioskop Microscope (Carl Zeiss, Oberkochen, Germany).

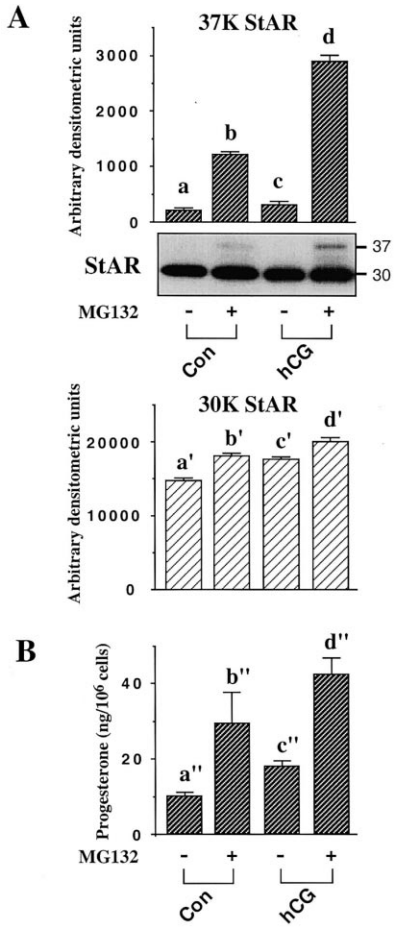

Fig. 2. A: StAR content by Western blot in preovulatory granulosa cells incubated at 37°C for 7 h in the absence or presence of hCG (3 IU/ml), and in the absence or presence of MG132 (10 μM). Densitometric tracing of the 37 kDa and 30 kDa StAR in the different treatments is shown above or below the blot, respectively. B: Progesterone secretion from preovulatory granulosa cells incubated at 37°C for 7 h in the absence or presence of hCG (3 IU/ml). Data are the mean ± S.D. ($n = 3$). a is different from c ($P < 0.05$). a′ is different from c′ ($P < 0.01$), and a″ is different from c″ ($P < 0.005$). a, c are different from b, d, respectively ($P < 0.001$). a′, c′ are different from b′, d′, respectively ($P < 0.05$). a″, c″ are different from b″, d″, respectively ($P < 0.001$).

2.7. Statistical analysis

Analysis of progesterone production and densitometer tracing on Western blot autoradiograms (mean ± S.D.) were performed using unpaired Student's *t* test and differences were significant if *P* values were < 0.05.

3. Results

In order to examine whether a specific proteasome inhibitor MG132 [18] can affect the intracellular level of StAR, immortalized human granulosa cells and preovulatory rat granulosa cells were incubated for 5–7 h with 10–30 μM of MG132 in the presence or absence of 25 μM of FK. There was a pronounced elevation in the 30 kDa and 37 kDa StAR proteins in immortalized human granulosa cells treated with MG132 in the absence of FK, but the augmentation was much more dramatic when cells were stimulated with FK (Fig. 1A). In contrast no elevation in 30 kDa and 37 kDa StAR protein was observed either in the presence or absence of FK upon incubation of the cells with 10 μM of an inhibitor of lysosomal proteases E-64 [19–21] (Fig. 1B). No elevation in 37 kDa StAR protein was evident even at 30 μM or 100 μM of E-64 (data not shown). An elevation in 30 kDa protein and a dramatic increase in 37 kDa protein was also evident in rFSHR-

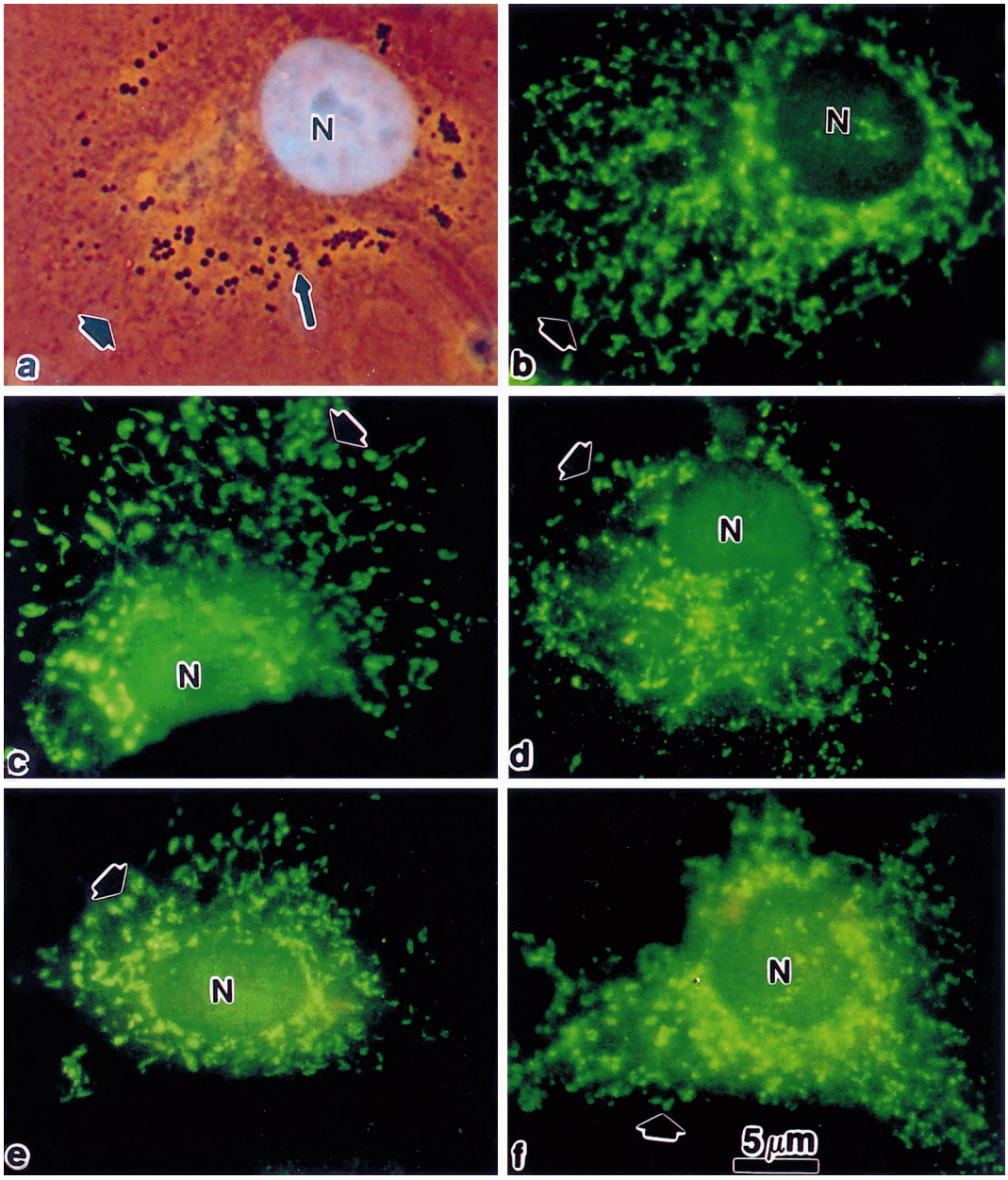

Fig. 3. Expression of StAR by immunocytochemistry in primary preovulatory granulosa cells treated with hCG in the absence or presence of a proteasome inhibitor MG132. Cells were incubated with 5% serum in the absence (a–c) or the presence (d) of 10 μM MG132 or with 3 IU/ml hCG in the absence (e) or presence (f) of MG132 for 5 h at 37°C. Cells were doubly stained (a, b) with DAPI (blue) (a) and anti-cytochrome *c* (green) (b) to visualize and to verify the location and the intactness of the mitochondria (dotted green fluorescence; wide arrows), lipid droplets, characteristic for steroidogenic cells (arrow) and the nucleus (N). Cells were stained with anti-StAR antibodies (c–f). Note localization of StAR in mitochondria (dotted green fluorescence) and some diffuse staining in cytoplasm in MG132-treated cells (d, f). a: Combined phase and fluorescent microscopy; b–f: fluorescent microscopy.

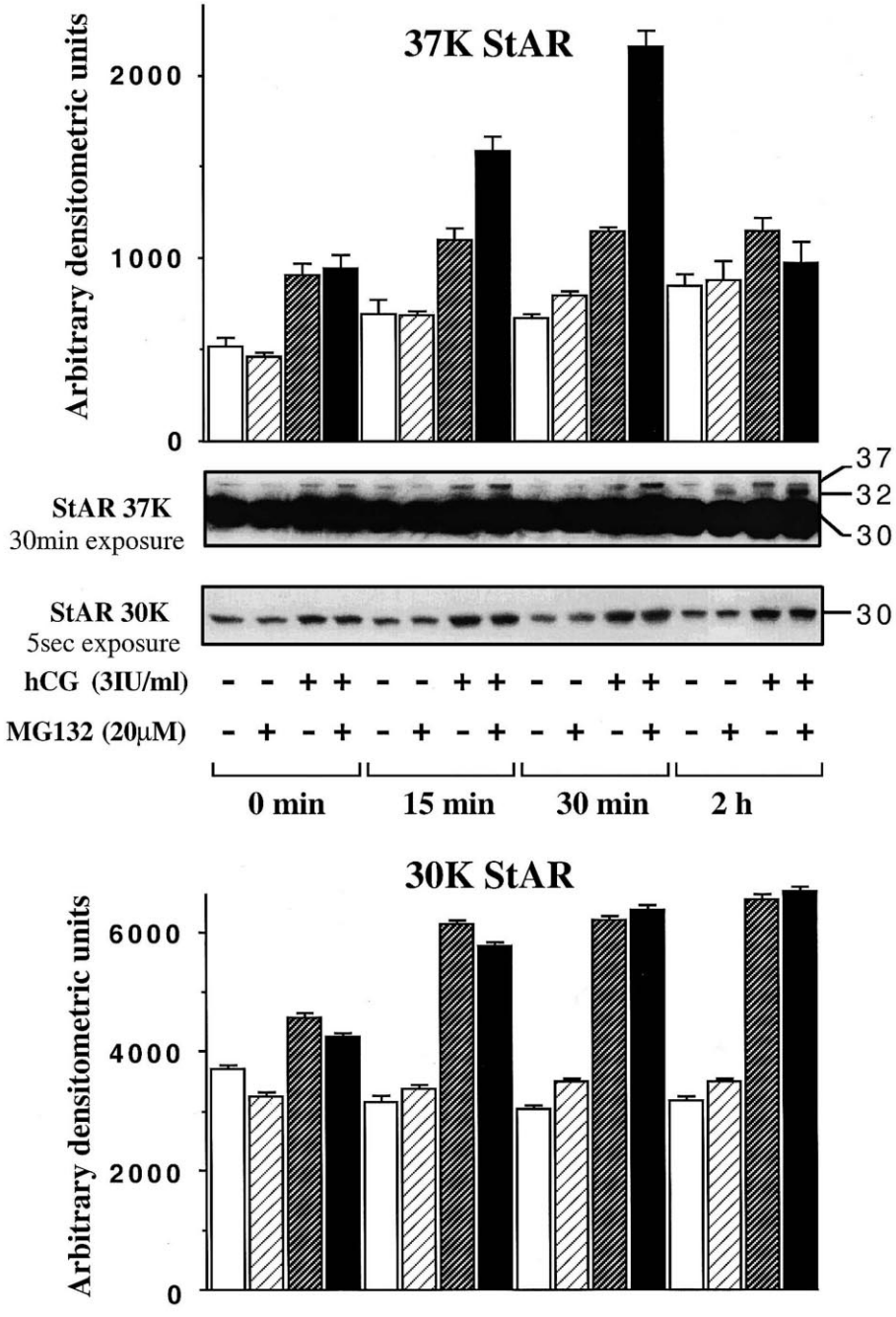

Fig. 4. Expression of StAR in preovulatory rat granulosa cells in the presence of a proteasome inhibitor MG132. Cells were isolated from PMSG-treated 25-day-old female rats and cultured for 24 h in 5% FCS in the absence or presence of 3 IU/ml hCG, and subsequently with 20 μM MG132 for 15 min, 30 min, or 120 min. Cell lysates were electrophoresed, blotted and incubated with rabbit antiserum to StAR protein. At the top and bottom, densitometric tracing of the 37 kDa and 30 kDa protein, respectively.

17 cells treated with FSH and MG132 compared to FSH treatment alone (Fig. 1C). Moreover a similar phenomenon was observed in primary cultures of preovulatory rat granulosa cells in the basal state or when stimulated with hCG. The increase in 37 kDa protein was much more pronounced than increase in 30 kDa protein (Fig. 2A). MG132 treatment did not elevate levels of two other proteins that are involved in cholesterol metabolism; the 14 kDa ADX protein, a component of the mitochondrial cholesterol side-chain cleavage system, and the 146 kDa NPC1 protein, a late endosome component that plays a central role in the trafficking of free cholesterol out of lysosomes (Fig. 1A). On the contrary some decrease of NPC1 levels was observed in the presence of FK and MG132.

In order to examine whether elevation of StAR content by MG132 enhances steroidogenesis we measured progesterone production in response to the various treatments. A significant elevation in progesterone formation was evident in MG132-treated cells (Fig. 2B). In order to determine whether the StAR protein is localized in the mitochondria in MG132-treated cells we immunostained cells with StAR antibody. Indeed, StAR was found to be localized in mitochondria of the

cells. Interestingly some elevation of cytoplasmic StAR was evident in cells treated with MG132 without or with hCG, which presumably reflects accumulation of the 37 kDa protein (Fig. 3).

In order to examine the rate of accumulation of StAR subsequent to MG132 treatment we preincubated preovulatory rat granulosa cells for 12 h in the presence or absence of hCG (3 IU/ml) followed by incubation with and without MG132 for 15 min until 8 h. Before the addition of MG132 there was elevated content of 37 kDa protein in hCG-treated cells, and there was additional 43% increase in 37 kDa protein in hCG+MG132-treated cells at 15 min compared to cells treated with hCG alone (Fig. 4). Further increases of 37 kDa protein in MG132-treated cells preincubated with hCG (187%) were evident 30 min following MG132 treatment. Increasing levels of 32 kDa protein were evident in MG132-treated cells for 1–2 h either preincubated or not with hCG, while modest increases in 30 kDa protein were evident only following 7 h of incubation with MG132 (Fig. 2).

4. Discussion

StAR protein plays a key role in rapid modulation of steroidogenesis in the ovary [22], testis [23] and the adrenal [24]. It has previously been demonstrated that expression of StAR is increased via a gonadotropin–cAMP–protein kinase A cascade involving steroidogenic factor 1 [25,26]. Interestingly, StAR expression is also independently elevated by growth factors such as basic fibroblastic growth factor [27] without the elevation of intracellular cAMP levels. In the immortalized granulosa cell line established in our laboratory [11,12] progesterone production declines within several hours after removal of the stimulant (FSH or FK, Sasson and Amsterdam, unpublished). This is consistent with a rapid loss of StAR functional activity.

In the present work we demonstrate that the 37 kDa precursor and to a lesser extent the 30 kDa mature protein accumulate upon inhibition of the proteasome, a multicatalytic protease [28] that degrades intracellular proteins that must be turned over rapidly. The preferential elevation of StAR without increases in NPC1 and the cytochrome P450scc enzyme system component, ADX, was associated with increased progesterone production, consistent with the notion that StAR regulation is the limiting step in steroidogenesis. Our studies demonstrate that the 37 kDa StAR protein is elevated within 15 min of exposure of both primary and immortalized granulosa cells to MG132. These observations are consistent with pulse-chase experiments where a clear degradation of the protein was also evident within a similar time frame [3]. Subsequent accumulation of 32 kDa protein can be explained by the accumulation of 37 kDa protein which is subsequently imported into mitochondria and processed to the 32 kDa and mature 30 kDa forms.

Only modest accumulation of the 30 kDa protein was observed in primary preovulatory granulosa cells, which have abundant 30 kDa StAR protein in the basal state. In contrast, in the immortalized HO-23 human granulosa cells which contain extremely low levels of StAR in non-stimulated state, accumulation of 30 kDa protein was evident within 5 h of incubation with MG132 and both 30 kDa and 37 kDa proteins were elevated when cells were pre-stimulated with FK for 10 h. The inability of MG132 to elevate intracellular levels of ADX or NPC1, proteins involved in cholesterol side-chain cleavage and intracellular cholesterol trafficking, respectively, which do not have short half-lives, contrasts with the effects of MG132 on StAR. This is concordant with the unique requirements for acute modulation of the rate-limiting step in steroidogenesis. Moreover our data strengthen the notion that the 37 kDa protein is the form of StAR that promotes translocation of cholesterol into the mitochondria because of the tight relationship between its accumulation and enhanced steroidogenesis that was demonstrated in this work.

Because of the relative low amount of 37 kDa StAR and the poor antibodies to ubiquitin, we were not able in preliminary experiments to demonstrate that the 37 kDa StAR is ubiquitinated prior to its degradation [29]. However, the inability of the calpain inhibitor E-64 to elevate the intracellular levels of StAR would strengthen our argumentation of the responsibility of the proteasome system for the degradation of StAR. Experiments designed to over-express StAR and labeled ubiquitin may clarify this issue in the future. Thus, at the present time we cannot rule out the possibility that StAR can be degraded by proteasomes without ubiquitination, which may be characteristic for proteins which are very rapidly degraded like ornithine decarboxylase [30].

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References

[1] Stocco, D.M. and Clark, B.J. (1996) Endocr. Rev. 17, 221–244.
[2] Strauss III, J.F., Kallen, C.B., Christenson, L.K., Watari, H., Devoto, L., Arakane, F., Kiriakidou, M. and Sugawara, T. (1999) Recent Prog. Horm. Res. 54, 369–395.
[3] Arakane, F., Kallen, C.B., Watari, H., Foster, J.A., Sepuri, N.B., Pain, D., Stayrook, S.E., Lewis, M., Gerton, G.L. and Strauss III, J.F. (1998) J. Biol. Chem. 273, 16339–16345.
[4] Selvaraj, N., Israeli, D. and Amsterdam, A. (1996) Mol. Cell. Endocrinol. 123, 171–177.
[5] Orian, A., Gonen, H., Bercovich, B., Fajerman, I., Eytan, E., Israel, A., Mercurio, F., Iwai, K., Schwartz, A.L. and Ciechanover, A. (2000) EMBO J. 19, 2580–2591.
[6] Oren, M. (1999) J. Biol. Chem. 274, 36031–36034.
[7] Bercovich, Z. and Kahana, C. (1993) Eur. J. Biochem. 213, 205–210.
[8] Leibovitz, D., Koch, Y., Fridkin, M., Pitzer, F., Zwickl, P., Dantes, A., Baumeister, W. and Amsterdam, A. (1995) J. Biol. Chem. 270, 11029–11032.
[9] Sutovsky, P., Moreno, R.D., Ramalho-Santos, J., Dominko, T., Simerly, C. and Schatten, G. (2000) Biol. Reprod. 63, 582–590.
[10] Amsterdam, A., Nimrod, A., Lamprecht, S.A., Burstein, Y. and Lindner, H.R. (1979) Am. J. Physiol. 236, E129–E138.
[11] Hosokawa, K., Dantes, A., Schere-Levy, C., Barash, A., Yoshida, Y., Kotsuji, F., Vlodavsky, I. and Amsterdam, A. (1998) Endocrinology 139, 4679–4687.
[12] Keren-Tal, I., Dantes, A., Sprengel, R. and Amsterdam, A. (1993) Mol. Cell. Endocrinol. 95, R1–R10.
[13] Kohen, F., Bauminger, S. and Lindner, H.R. (1975) in: Steroid Immunoassay (Cameron, E.H.D., Hillier, S.G. and Griffiths, K., Eds.), pp. 11–23, Alpha Omega Publishing Ltd., Cardiff.
[14] Keren-Tal, I., Dantes, A. and Amsterdam, A. (1996) Mol. Cell. Endocrinol. 116, 39–48.
[15] Bradfold, M.M. (1976) Anal. Biochem. 72, 248–254.
[16] Liscum, L. and Underwood, K.W. (1995) J. Biol. Chem. 270, 15443–15446.
[17] Watari, H., Blanchette-Mackie, E.J., Dwyer, N.K., Watari, M.,

Neufeld, E.B., Patel, S., Pentchev, P.G. and Strauss, J.F. (1999) J. Biol. Chem. 274, 21861–21866.
[18] Lee, D.H. and Goldberg, A.L. (1996) J. Biol. Chem. 271, 27280–27284.
[19] Hanada, K., Tamai, M., Yamagishi, M., Ohmura, S., Sawada, J. and Tanaka, I. (1978) Agric. Biol. Chem. 42, 523–528.
[20] Barrett, A.J., Kembhavi, A.A., Brown, M.A., Kirschke, H., Knight, C.G., Tamai, M. and Hanada, K. (1982) Biochem. J. 201, 189–198.
[21] Sarin, A., Clerici, M., Blatt, S.P., Hendrix, C.W., Shearer, G.M. and Henkart, P.A. (1994) J. Immunol. 153, 862–872.
[22] Sugawara, T., Holt, J.A., Driscoll, D., Strauss III, J.F., Lin, D., Miller, W.L., Patterson, D., Clancy, K.P., Hart, I.M., Clark, B.J. and Stocco, D.M. (1995) Proc. Natl. Acad. Sci. USA 92, 4778–4782.
[23] Gradi, A., Tang-Wai, R., McBride, H.M., Chu, L.L., Shore, G.C. and Pelletier, J. (1995) Biochim. Biophys. Acta 1258, 228–233.
[24] Liu, J., Heikkila, P., Kahri, A.I. and Voutilainen, R. (1996) J. Endocrinol. 150, 43–50.
[25] Sugawara, T., Holt, J.A., Kiriakidou, M. and Strauss, J.F. (1996) Biochemistry 35, 9052–9059.
[26] Caron, K.M., Ikeda, Y., Soo, S.C., Stocco, D.M., Parker, K.L. and Clark, B.J. (1997) Mol. Endocrinol. 11, 138–147.
[27] Aharoni, D., Meiri, I., Atzmon, R., Vlodavsky, I. and Amsterdam, A. (1997) Curr. Biol. 7, 43–51.
[28] Lee, D.H. and Goldberg, A.L. (1998) Trends Cell Biol. 8, 397–403.
[29] Hershko, A. and Ciechanover, A. (1998) Annu. Rev. Biochem. 67, 425–479.
[30] Bercovich, Z., Rosenberg-Hasson, Y., Ciechanover, A. and Kahana, C. (1989) J. Biol. Chem. 264, 15949–15952.